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ACTIVATION OF PHOSPHATIDIC ACID METABOLISM OF HUMAN ERYTHROCYTE MEMBRANES BY PERFRINGOLYSIN O

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The effect of perfringolysin O on the lipid metabolism of human erythrocyte membranes was investigated. Erythrocytes were prelabeled with [^{3}H]arachidonic acid and [^{32}P]inorganic phosphate. In the presence of calcium ion(5.5 mM), the effect of perfringolysin O on lipid metabolism was very similar to that of an calcium-ionophore A23187. In the absence of calcium ion, the accumulation of phosphatidic acid and its following decreasing trend were observed during the reaction with the toxin. Such changes were not caused by filipin. These results suggest that perfringolysin O causes the activation of a diglyceride-phosphatidic acid cycle, which might be involved in the calcium transport. © 1986 Academic Press, Inc.

Perfringolysin O is an exotoxin produced by Clostridium perfringens, type A, and is known as a highly potent hemolysin(1,2). It is a member of "thiol-activated cytolysins", which share basic characteristics in their biological reactions such as specific binding to cholesterol, non-colloid-osmotic and temperature-dependent hemolysis(2). Although some properties of the toxins and their hemolysis reactions have been reported, only limited information is available with regard to changes in cell membranes caused by the toxins(3,4). In an earlier report, we showed that perfringolysin O markedly accelerates the permeability of human erythrocyte membranes to

Abbreviation used: HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid.

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calcium ion(5). This finding led us to an idea that acidic phospholipid metabolism might be activated by the toxin to increase the calcium permeability, since acidic lipids, especially phosphatidic acid, are supposed to be involved in the calcium transport phenomenon of cell membranes(6). In the present study, we show evidence suggesting that perfringolysin O causes the activation of phosphatidic acid metabolism of human erythrocyte membranes.

Materials and Methods

Perfringolysin O was purified as reported previously(7). The specific activity of the purified toxin, which was activated by 10 mM dithiothreitol, was 4.6×10^7 hemolytic units/mg protein, where one hemolytic unit was defined as a toxin amount required to cause 50% hemolysis of sheep erythrocytes(0.5% suspension, 0.5 ml) at 37° for 30 min(7). The inactivated toxin was prepared by incubating the toxin(800 hemolytic units) with cholesterol(50 μg) for 15min at 37°.

Membrane lipids of human erythrocytes were labeled with radioisotope precursors as follows. Erythrocytes were suspended at the concentration of 50% in 5 ml of 0.9% NaCl-10 mM HEPES(NaCl-HEPES buffer, pH 7.30) containing 1 mg/ml glucose, and incubated with 250 μCi of [^{3}H]arachidonic acid(100 Ci/mmol, New England Nuclear) and 1 mCi of [^{32}P]inorganic phosphate(carrier-free, New England Nuclear) for two hours at 37°. The labeled erythrocytes were washed three times with the NaCl-HEPES buffer and resuspended in the same buffer.

Aliquots of the suspension(42%, 0.08 ml) were incubated with 0.02 ml of the activated toxin solution(17 hemolytic units dissolved in the NaCl-HEPES buffer) and 0.12 ml of the NaCl-HEPES buffer containing 10 mM $CaCl_2$ at 37° for 1, 2, 3, 5 and 10 min. For control, $CaCl_2$ was omitted from the reaction mixture. Lipids extracted with 2 ml of chloroform/methanol(1/1, v/v) were separated by two dimensional thin-layer chromatography(8), and the radioactivity corresponding to each spot of lipids was measured. A labeled erythrocyte suspension(25%, 0.2 ml) was also incubated with 0.02 ml of the activated(17 hemolytic units) or inactivated toxin solution at 37° up to 10 min.

The hemolysis rection with filipin was carried out by incubating a labeled erythrocyte suspension(25%, 0.16 ml) with 0.09 ml of the NaCl-HEPES buffer containing 6.75 μg filipin(Upjohn). Labeled erythrocytes(15%, 0.4 ml) were also incubated with 5 μl of 5 mM A23187(in ethanol, Boeringer-Mannheim) and 0.1 ml of the NaCl-HEPES buffer containing 25 mM $CaCl_2$ at 37°.

Results and Discussion

When erythrocytes were incubated with [^{3}H]arachidonic acid and [^{32}P]inorganic phosphate, the [^{3}H]-radioactivity appeared in all phospholipids and diglyceride, while the [^{32}P]-radioactivity was exclusively incorporated into phosphatidic acid. It is assumed that [^{32}P]phosphatidic acid was formed through phosphorylation of diglyceride with [^{32}P]-γ-ATP by the action of diglyceride kinase(9).

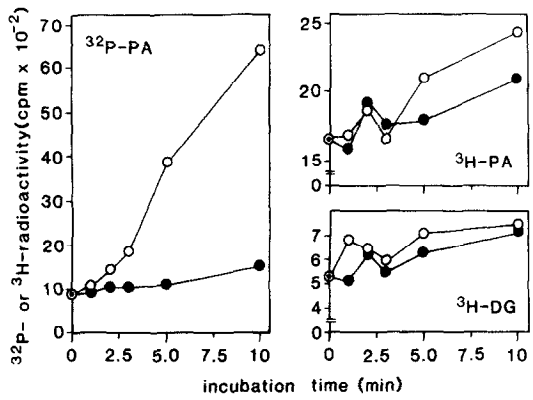

Fig.1. Effect of perfringolysin O on [^{32}P]phosphatidic acid(left), [^{3}H]phosphatidic acid(above right) and [^{3}H]diglyceride(below right) under 5.5 mM(-o-o-) or 0 mM(-●-●-) calcium ion. The figure is the result of a typical experiment(one of three similar experiments).

The labeled erythrocytes were treated with perfringolysin O under 5.5 mM calcium ion. The treatment with the toxin of labeled cells produced a marked increase of the [^{32}P]-radioactivity of phosphatidic acid, reaching at 10 min to the 4.2-fold level of that observed without calcium ion(Fig.1, left). The [^{3}H]-radioactivity of phosphatidic acid also increased during the reaction(Fig.1, right). These changes were very similar to those observed when labeled cells were treated with a calcium-ionophore A23187(Table 1). Since the changes observed with A23187 are assumed to be caused by calcium ion introduced into cells, the above observation supports our previous finding that perfringolysin O accelerates the calcium ion permeability of erythrocyte membranes(5). Allan and Michell(10,11) carried out similar experiments with A23187, and reported that the accumulation of diglyceride occured along with the depletion of intracellular [^{32}P]-γ-ATP

Table 1. Effect of perfringolysin O or A23187 on phosphatidic acid metabolism of human erythrocytes. Labeled cells were treated with either of the agents under 0 mM or 5.5 mM calcium ion at 37° for three min.

radioactivity(cpm)	(-)	Ca^{++}	toxin+Ca^{++}	A23187+Ca^{++}
[^{32}P]phosphatidic acid	321	342	1,149	1,135
[^{3}H]phosphatidic acid	536	515	881	807

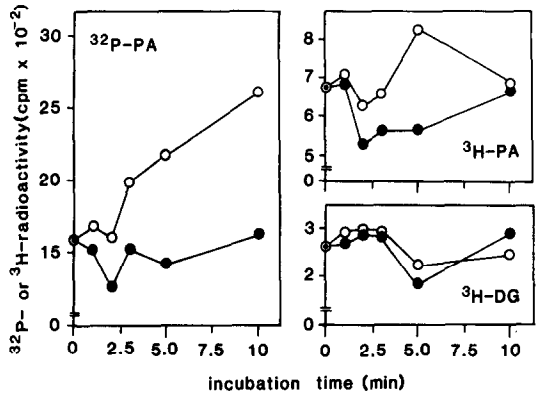

Fig.2. Effect of perfringolysin O on [^{32}P]phosphatidic acid(left), [^{3}H]phosphatidic acid(above right) and [^{3}H]diglyceride(below right) under no calcium ion. Labeled erythrocytes were treated with the activated toxin(-o-o-) or cholesterol-inactivated toxin(-●-●-).

after the period of active labeling with [H^{3}] of phosphatidic acid. When labeled cells were treated with perfringolysin O, no change in the [^{3}H]-radioactivity of diglyceride was observed(Fig.2, right), suggesting that intracellular ATP was preserved to be sufficient during the reaction. These results suggest that the changes observed with perfringolysin O and calcium ion are attributable mostly to the effect of calcium ion introduced by the toxin into cells.

In the absence of calcium ion, the [^{32}P]-radioactivity of phosphatidic acid increased during the reaction, and reached to the 1.6-fold level of the control at 10 min(36% hemolysis at 10 min)(Fig.2, left). Filipin, one of polyene antibiotics, is known to bind specifically to cholesterol to cause hemolysis like perfringolysin O(12). The treatment with filipin of labeled erythrocytes, however, did not produce any apparent changes in the [^{32}P]-radioactivity of phosphatidic acid(64% hemolysis at 10 min)(data not shown). Thus the activation of the phosphorylation process of diglyceride seems to be a specific effect of perfringolysin O.

The effect of perfringolysin O on [^{3}H]phosphatidic acid was different from that on [^{32}P]phosphatidic acid(Fig.2, right). The [^{3}H]-radioactivity of phosphatidic acid increased to 1.4-folds of the control at 5 min, and returned to the control level at 10 min. Since the early increase in the

[^{3}H]-radioactivity suggests that [H^3]phosphatidic acid was newly formed through phospharylation of [^{3}H]diglyceride, a reduction in the [^{3}H]-radioactivity of diglyceride would be expected to occur at the early phase of the reaction. However, the [^{3}H]-radioactivity of diglyceride did not show any difference from the control(Fig.2, right). This may imply that [^{3}H]diglyceride was further supplied from other sources. Since the chemical analysis of membrane lipids showed a 35% decrese in the content of phosphatidylinositol without apparent changes in those of other lipids(Saito,M. and Ando,S., unpublished data), it seems reasonable to assume that perfringolysin O causes the degradation of phosphatidylinositol to generate diglyceride. [H^3]-radioactivities of phosphatidylinositol as well as other phospholipids, however, did not apparently change during the reaction(data not shown). It is known that in human mature erythrocytes phosphatidylinositol is practically not synthesized from diglyceride, but can be formed through the degradation of polyphosphoinositides(13). In the case with perfringolysin O, phosphatidylinositol might further be supplied from polyphosphoinositides.

The subsequent decrease in the [^{3}H]-radioactivity of phosphatidic acid suggests that accumulated phosphatidic acid was degraded as the reaction proceeded. Phosphatidic acid is known to be degraded either by the action of phosphatidic acid phosphatase or phospholipase A in human mature erythrocytes(13). Since no appreciable increment of [^{3}H]-radioactivity in free fatty acids was observed(data not shown), phospholipase A might not be activated. Phosphatidic acid phophatase seems to be rather relevant to the degradation of phosphatidic acid to diglyceride. Diglyceride formed may be converted to phosphatidic acid again by the action of diglyceride kinase. It thus is suggested that the dephosphorylation process might be initiated as the accumulation of phosphatidic acid proceeds, resulting in the activation of a diglyceride-phosphatidic acid cycle.

It is not known at present whether the activation of the phosphatidic acid metabolism is involved in the acceleration of the calcium ion transport

by perfringolysin O. Since the increase in the [^{3}H]-radioactivity of phosphatidic acid was transient and of low degrees in the absence of calcium ion, it seems questionable that the amount of phosphatidic acid accumulated is enough to function as a calcium ionophore. When fluoride ion(10 mM), which is an inhibitor for phosphatidic acid phosphatase, was introduced into resealed ghosts of human erythrocytes, the lysis of the ghosts by the toxin was strongly inhibited, while the lysis by phospholipase C(2.5 mU/ml, from Clostridium perfringens, Sigma) was not affected. This may suggest that the dephosphorylation process of phosphatidic acid is essential to hemolysis reaction by perfringolysin O. The hemolysis of human erythrocytes was induced when intracellular calcium ion of the cells was depleted with A23187(5). Thus the activation of the diglyceride-phosphatidic acid cycle is supposed to cause the accerelation of the calcium transport, resulting in the depletion of intracellular calcium ion.

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